Identification of NF-κB Inhibitors from Watercress

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for Graduation

with Distinction and Honors in Bachelor of Science of Pharmaceutical Sciences

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by

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Acknowledgments

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Abstract

Watercress, *Nasturtium officinale* W.T. Aiton, a member of the Brassicaceae family[1], is highly consumed in salad mixes and contains many phytochemicals[2]. As part of the cancer chemoprevention drug discovery program, watercress was tested in a panel of bioassays. The small scale hexane extract of watercress exhibited over 65% NF-kB inhibition at a concentration of 50 µM. NF-κB is a hallmark for cancer and disregulation of NF-κB has also been linked to inflammation and auto immune diseases[3]. Three isolated samples from the hexane extract were tested in assays for biological activity. While the large scale hexane extract of watercress did not demonstrate potent NF-κB inhibition, it was found that one of the isolated samples showed some activity in the NF-κB assay.

Introduction

NF-κB and Cancer

Nuclear factor-κ B (NF-κB) is a transcription factor that plays an important role in inflammation and proliferation of cells[3]. The activation of the NF-κB cell signaling pathway is responsible for survival signaling, proliferative control, and moreover promotes the resistance to certain tumorigenic agents and chemotherapy[4]. NF-κB prevents cancer cells from entering apoptosis and contributes to cancer progression in certain cancers[5]. Elevated NF-κB activity has been observed in many cancers[6] and is found activated in the nucleus of certain types of cancer cells[7]. Recent studies have linked deviant activation of NF-κB with many different types of cancers[8]. Other studies have found that suppression of NF-κB limits the replication of cancer cells[9]. This characteristic of NF-κB makes it an attractive target for research that aims to inhibit the growth of cancer cells. Many natural metabolites that have shown anticancer activity also show inhibition of NF-κB, and extracts from many dietary plants are efficient inhibitors of NF-κB activation *in vitro*[9].

Watercress and Cancer

Watercress, a green leafy, edible vegetable of the Brassicaceae family[1], has been found to contain many phytochemicals(see figure 1)[2]. A phytochemical, sometimes called a phytonutrient, can be one of many bioactive chemical compounds found in a plant that is proven to be beneficial to health[2]. Watercress contains oils, like mustard oil[10]. Watercress and other Brassica vegetables contain isothiocyanates such as phenyl isothiocyanate that have

Figure 1. Image of watercress leaves[1].

cancer-preventive qualities[11]. Glucosinolates like gluconasturtiin and carotenoids like lutein are also found in watercress[12]. A high dietary intake of watercress has been linked to a reduction in cancer[13] and reduces the DNA damage in white blood cells and increases the antioxidant uptake[14]. The crude extract of watercress has demonstrated significant anti-genotoxic, anti-proliferative and anti-metastatic potential in certain cancer cells[15]. This research project investigates the NF-κB inhibitory potential of metabolites isolated from the hexane extract of watercress.

Hypothesis and Objectives

The main objective of this investigation is to identify the NF- *κ*B inhibitors found in watercress. This investigation can provide new NF- *κ*B inhibitors that can be used in cancer chemoprevention treatment. Furthermore, the newly found inhibitors will lead scientists to better understanding the mechanisms of NF- *κ*B inhibition by watercress and its bioactive metabolites. It is hypothesized that the hexane extract of watercress will consistently demonstrate NF- κB inhibition and compounds isolated from the hexane extract will also demonstrate NF- κB inhibition.

Objective 1: To screen *Nasturtium officinale* for cancer chemopreventive properties in a panel of bioassays.

Objective 2: To isolate compounds from *Nasturtium officinale* that demonstrate NF-kB inhibitory activity.

Materials and Methods

Extraction, isolation and purification of compounds isolated from watercress were performed as presented in figure 2.

Extraction: Watercress (50 g) was macerated and extracted with ethanol 95%. The ethanol extract was dried using a rotary evaporator. An aliquot of this crude extract was submitted for testing in the NF- κB assay.

Partition and isolation: The crude extract was partitioned with ethyl acetate, hexane and water using a separator funnel, and then again prepared and submitted for testing in the NF-κB assay. Using the results from the small scale testing, extraction and partition was again performed with 1 kg watercress (sample purchased at Giant Eagle 2845 N. High Street, Columbus Ohio 43202). The most active partition from the preliminary screening, the hexane extract, was submitted to flash column chromatography using silica gel (Whatman 60A silica gel; mesh size 70-230) and reverse-phase column chromatography using C_{18} silica gel. Polarity was slowly increased; the solvents used in increasing concentrations were hexane, ethyl acetate, and methanol. Solutes lower in polarity eluted out of the column first followed by the more polar compounds.

Purification: Three compounds were purified and separated from a fraction of the hexane column (isolated at a low polarity of 1:9 ethyl acetate: hexanes) using preparative thin-layer chromatography (prep-TLC; Whatman 60A silica gel) with dimensions 20X20 cm with a thickness of 200 µm. For analysis, the TLC plates were developed with

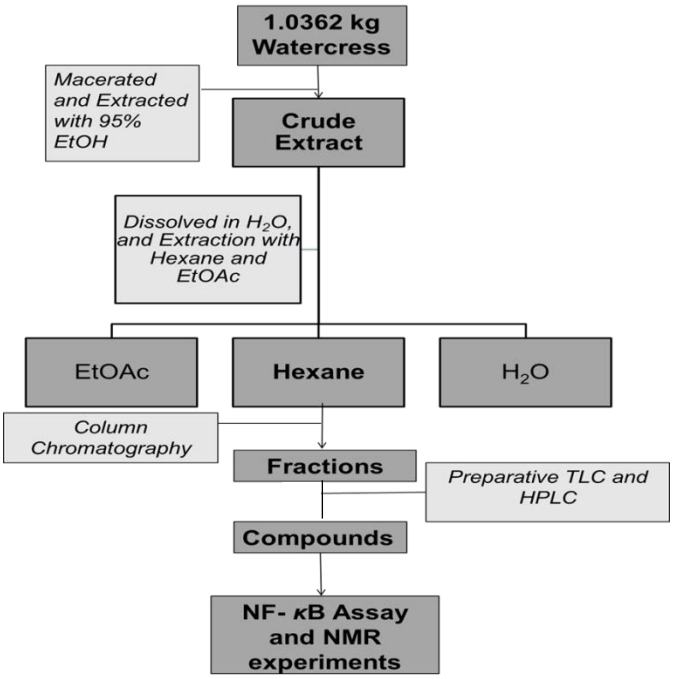


Figure 2. Large Scale Isolation Scheme.

phosphoric acid and heated at 110°C on a hot plate. High-performance liquid chromatography (HPLC) was also used for purification using an HPLC system (Waters™ 600 Controller and Waters™ 717 plus Autosampler) that includes pumps and a photodiode array detector (Waters™ Photodiode 996 Array Detector). The column used was Agilent Prep C_{18} Scalar with the dimensions 4.6 X 250 mm (10 µm). Both isocratic and gradient runs were performed on the compounds, using the solvents acetonitrile and water at varying percentages.

Identification: The compounds were identified using a combination of spectroscopic techniques such as ultraviolet light (UV), mass spectrometry (MS), and nuclear magnetic resonance (NMR). Two compounds were sent to the Mass Spectrometry and Proteomics Facility, Campus Chemical Instrument Center for MS testing. The NMR experiments (Bruker-Spectrospin 400 MHz) performed were 1D-NMR, 2D-NMR, COSY, HMBC, HSQC and NOESY.

Biological testing: Extracts and partitions were submitted for testing in the NF- *κ*B and K-ras assays available in the laboratory. Samples exhibited activity only in the NF-κB assay. The NF- *κ*B inhibitory assay (EZ-detect™ transcription factor assay system; Pierce Biotechnology, Rockford, IL, USA) was used for screening all watercress samples. In brief, cancer cells from the HeLa cell line were treated with active compounds and their nuclei were extracted. The binding ability of NF- *κ*B was then tested in the nucleus, which was measured by a chemiluminescent signal in a plate reader (FLUOstar Optima). If a compound is active in the NF- *κ*B assay, then the binding ability of the transcription factor should decrease. The assay was repeated with 50 g and 1 kg of watercress to confirm results. Rocaglamide was used as the positive control and an untreated cancer cell was used as the negative control[16, 17].

Results

Phytochemical Analysis

The table below shows the amounts of the three compounds obtained from watercress overall.

Table 1. Quantity of Each Compound Isolated via Preparatory-TLC

Compound	Amount submitted for NF-κB Bioassay(mg)	Amount Used in NMR Experiments (mg)	Total Amount (mg)
KW6	0.89	3.0	3.89
KW6-4	0.5	6.0	6.5
KW6-7	0.5	6.4	6.9

Purity Evaluation via HPLC

The chromatograms below indicate the retention time of each compound along with relative purity.

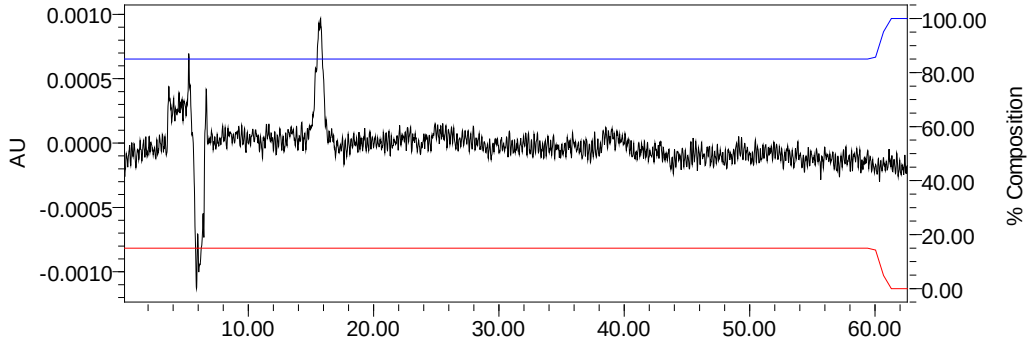


Figure 3. Isocratic chromatogram of KW6 at a wavelength of 358 nm.
Amount injected = 5 µg
Solvent A(red) = water (15%-0%)
Solvent B(blue) = acetonitrile (85%-100%)
Retention time = 15.26 minutes
This chromatogram was not used as an indicator for purity, as the chromatogram indicates a dip around 6 minutes.

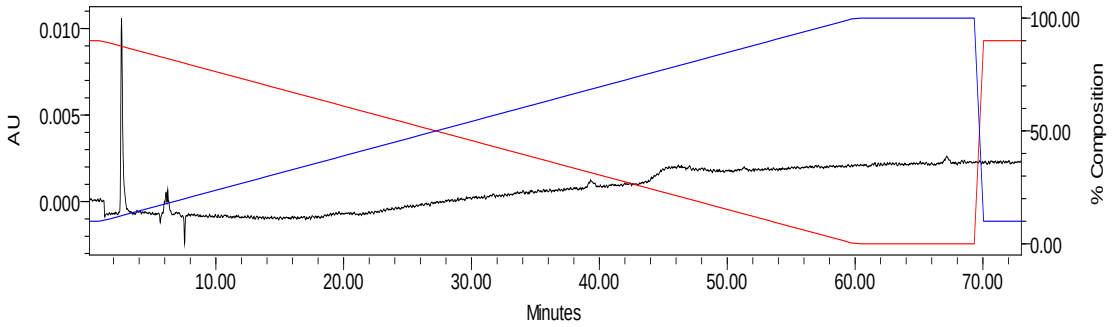


Figure 4. Gradient chromatogram of KW6 at a wavelength of 358 nm.
Amount injected = 5 µg
Solvent A(red) = water (90%-0%)
Solvent B(blue) = acetonitrile (10%-100%)
Retention time = 2.536 minutes
Lone peak suggests purity of compound.

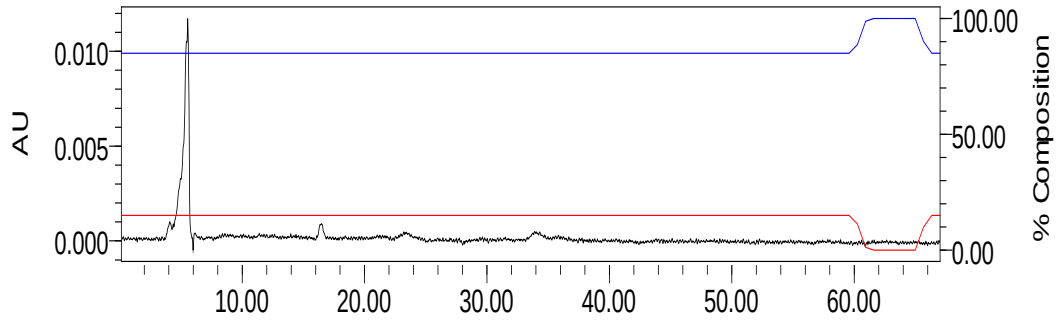


Figure 5. Chromatogram of KW6-4 at a wavelength of 358 nm.
Amount injected = 5 µg
Solvent A(red) = water (15%-0%)
Solvent B(blue) = acetonitrile (85%-100%)
Retention time = 5.132 minutes
Lone, single peak suggests purity of compound KW6-4.

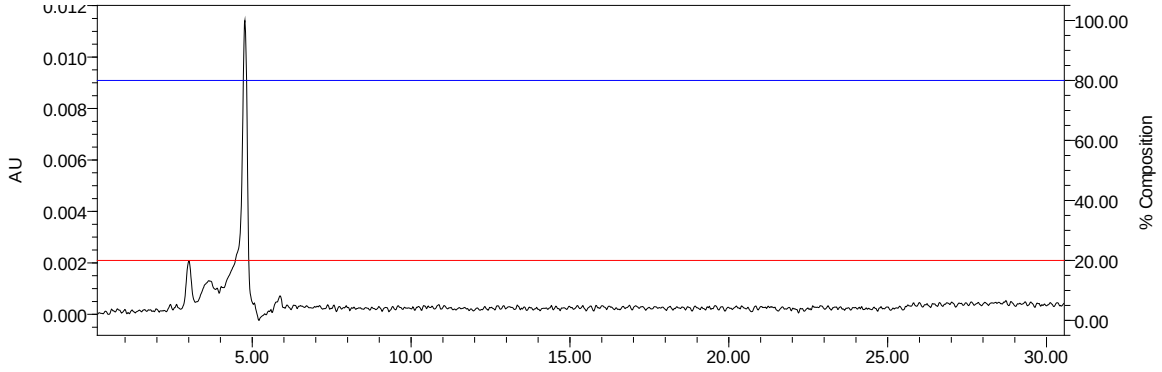


Figure 6. Isocratic chromatogram of KW6-7 at a wavelength of 358 nm.
Amount injected = 5 µg
Solvent A(red) = water (20%)
Solvent B(blue) = acetonitrile (80%)
Retention time = 4.775 minutes
Several small peaks attached to the large peak suggest impurities. This compound was not sent for mass spectrometry for this reason.

Compound identification via MS analysis

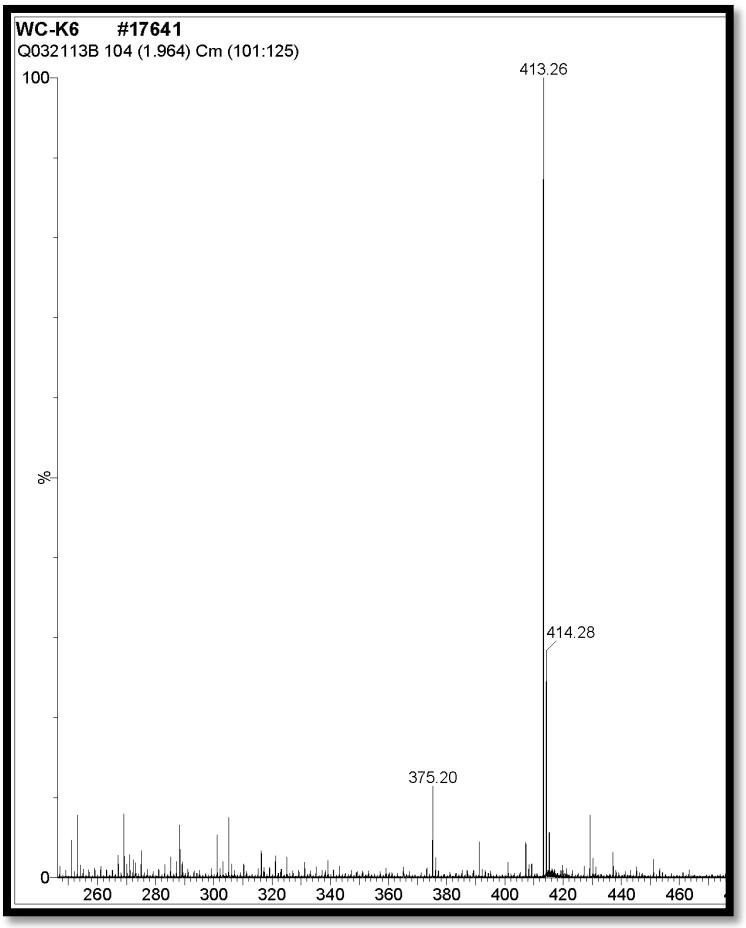


Figure 7. Mass Spectrum of KW6 (*m/z* 413.26).

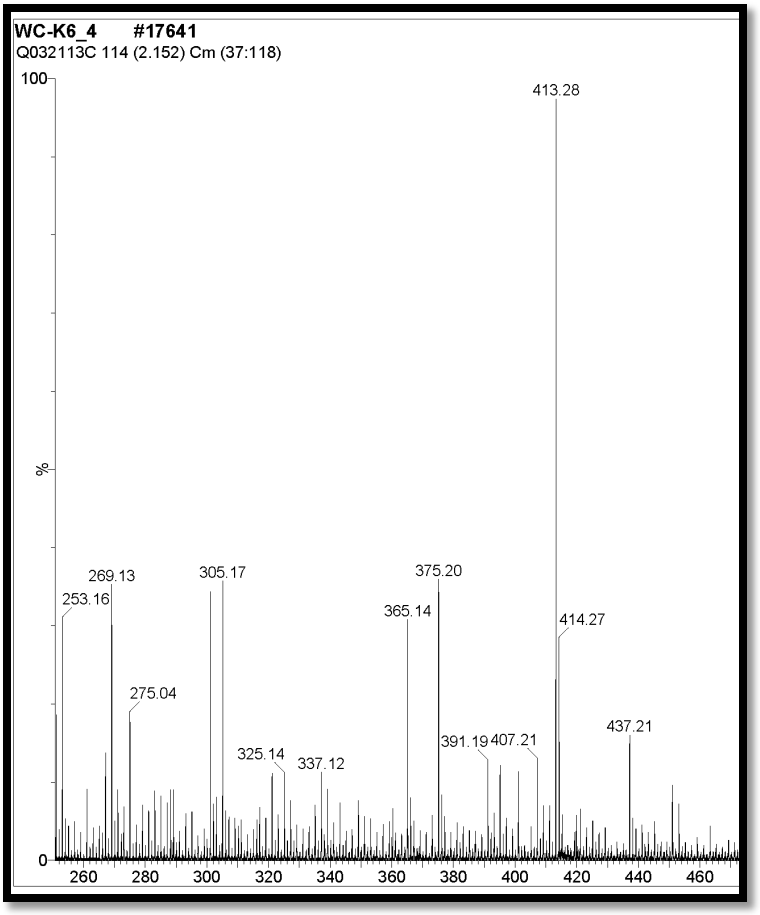


Figure 8. Mass Spectrum of KW6-4 (*m/z* = 413.28).

Compound identification via NMR data analysis

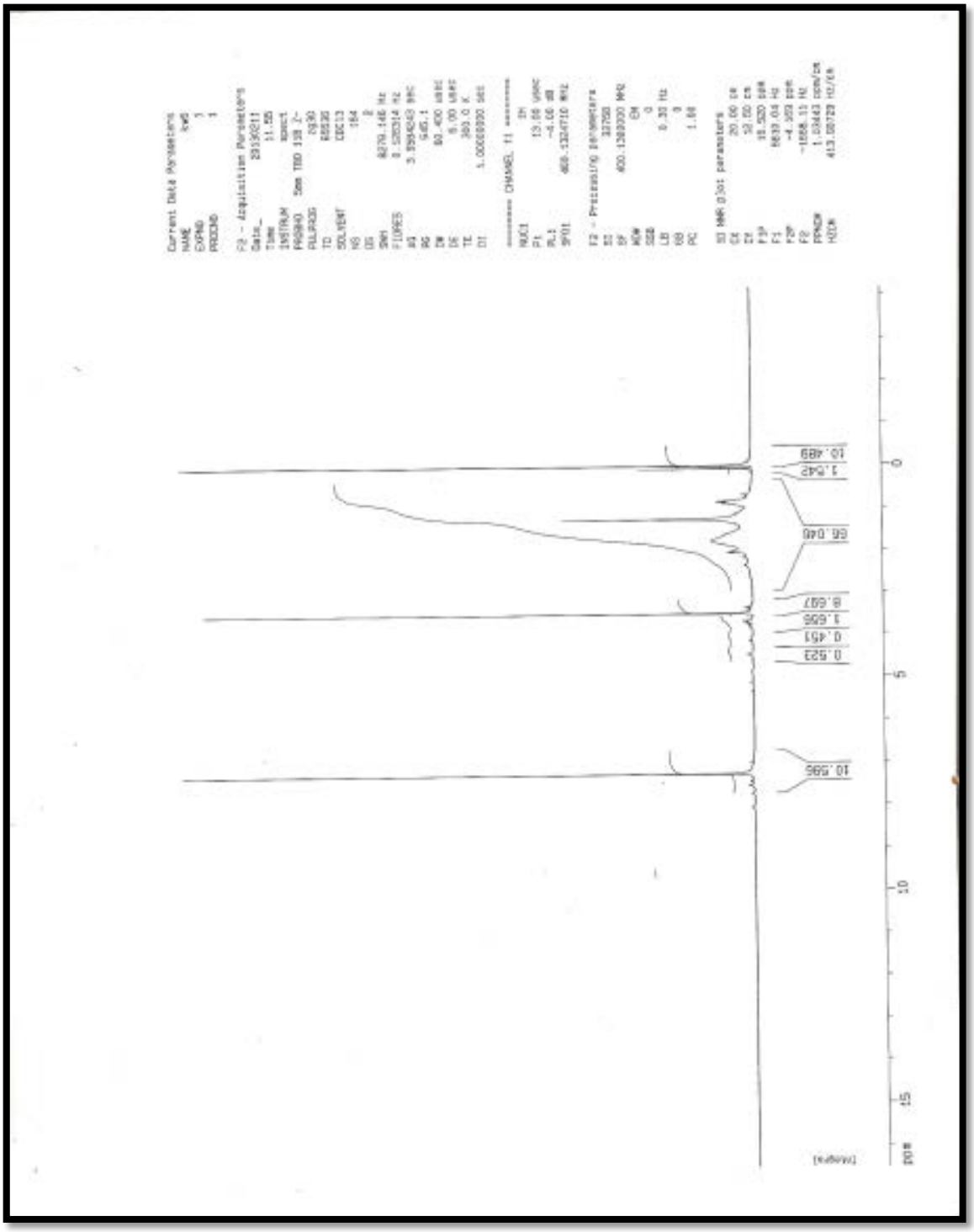


Figure 9. 1D ^{1}H-NMR spectrum of KW6 in $CDCl_3$.

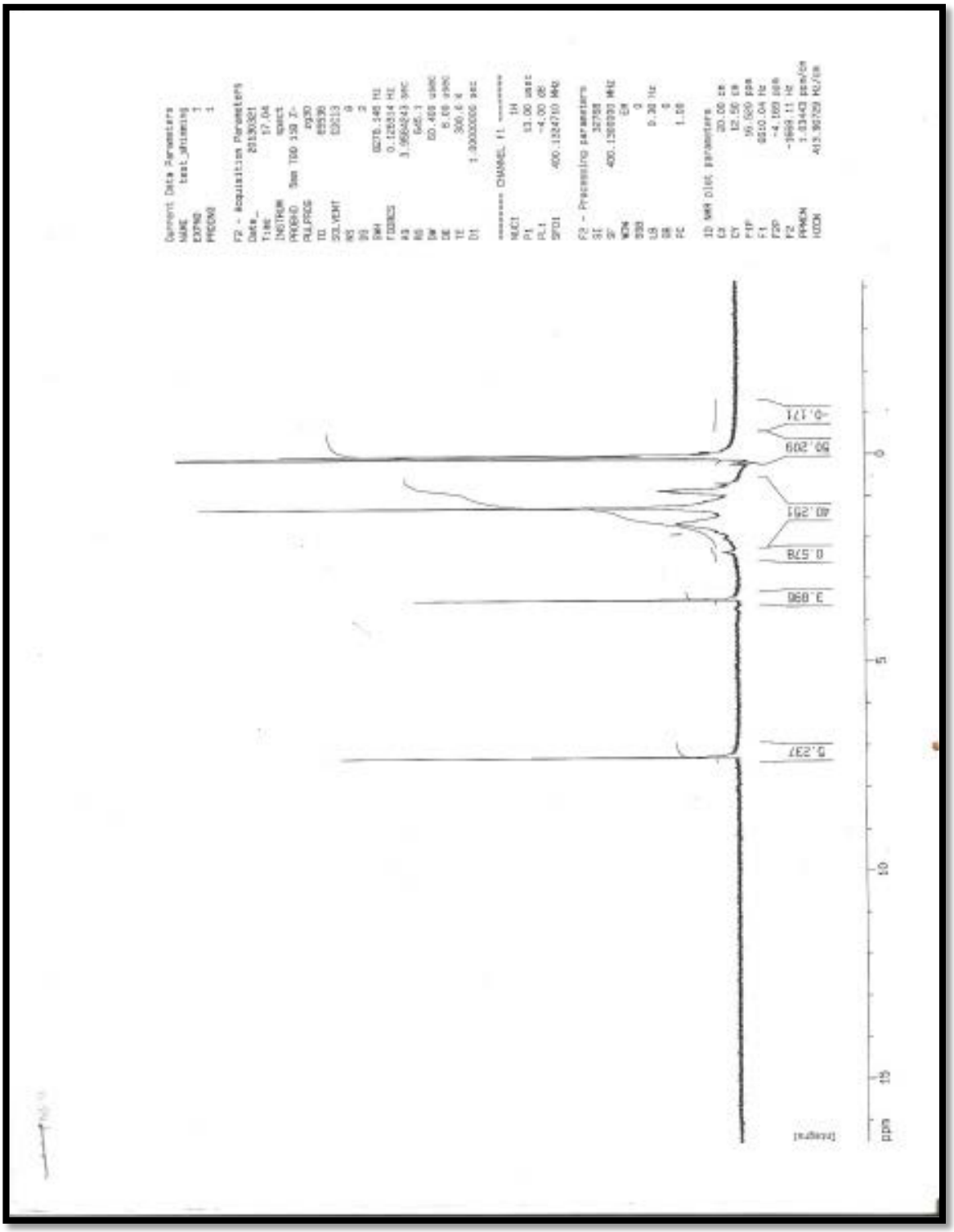


Figure 10. 1D ^{1}H-NMR spectrum of KW6-4 in $CDCl_3$.

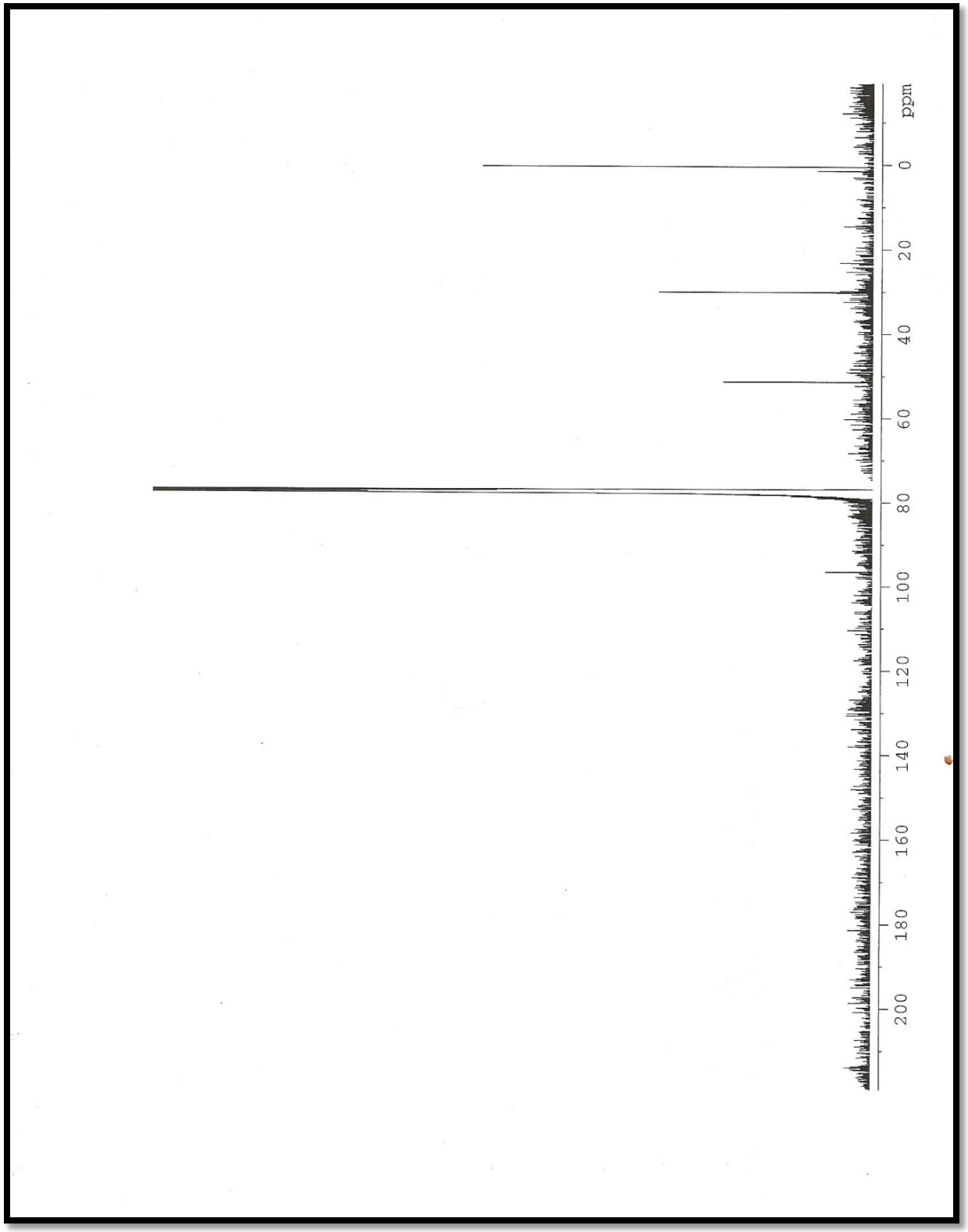


Figure 11. 1D ^{13}C-NMR spectrum of KW6 in $CDCl_3$.

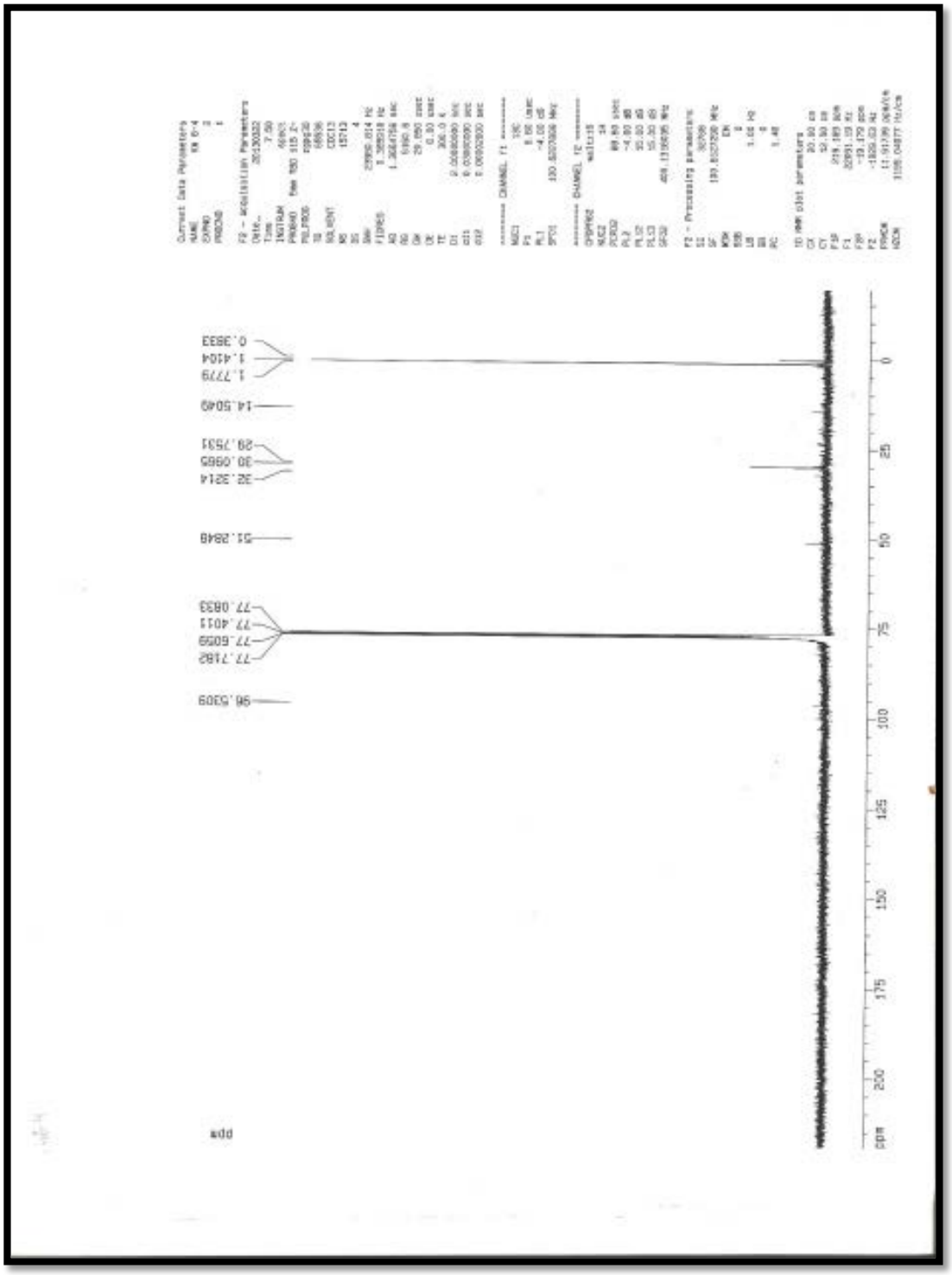


Figure 12. 1D ^{13}C-NMR spectrum of KW6-4 in $CDCl_3$.

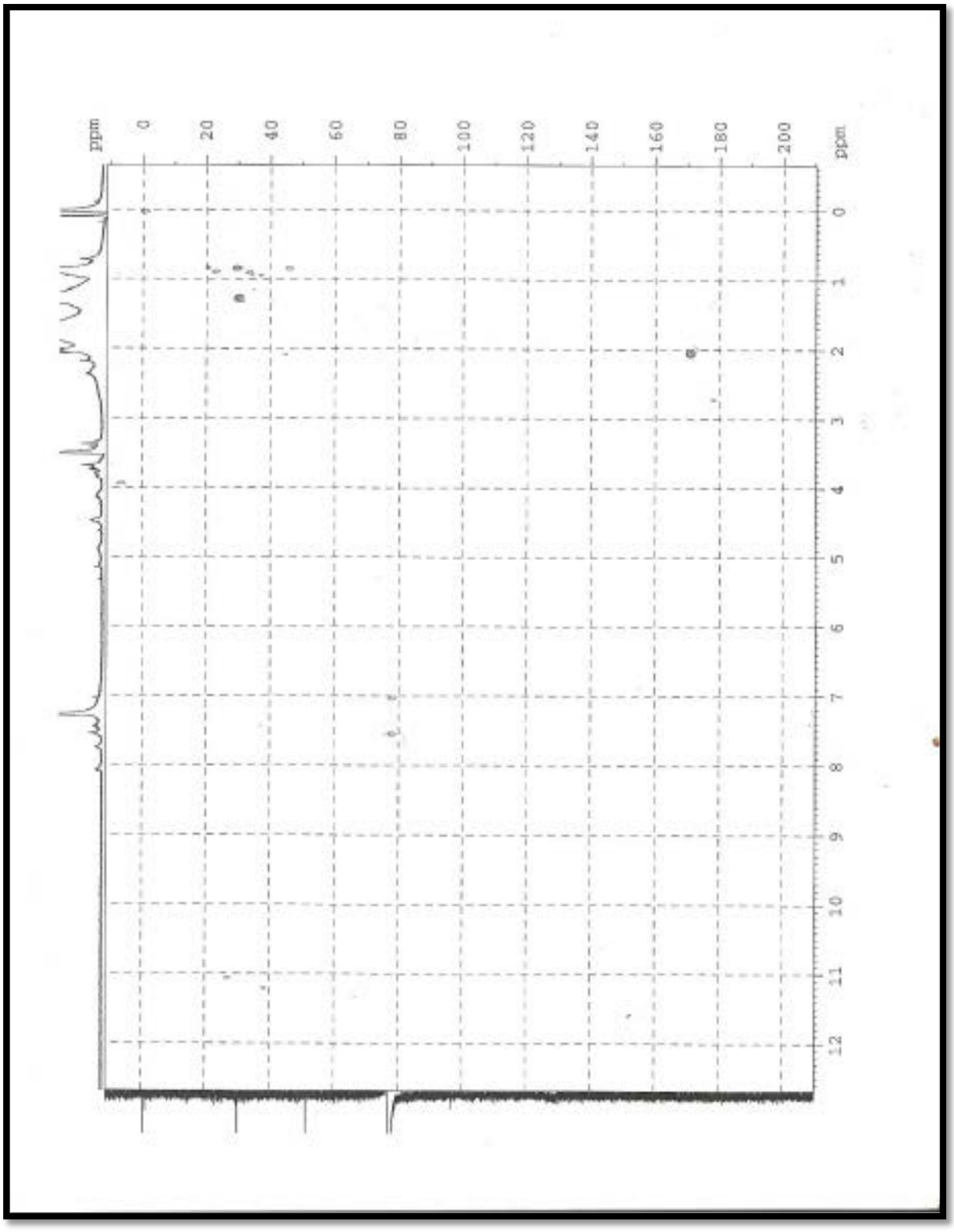


Figure 13. HMBC of KW6 in $CDCl_3$.

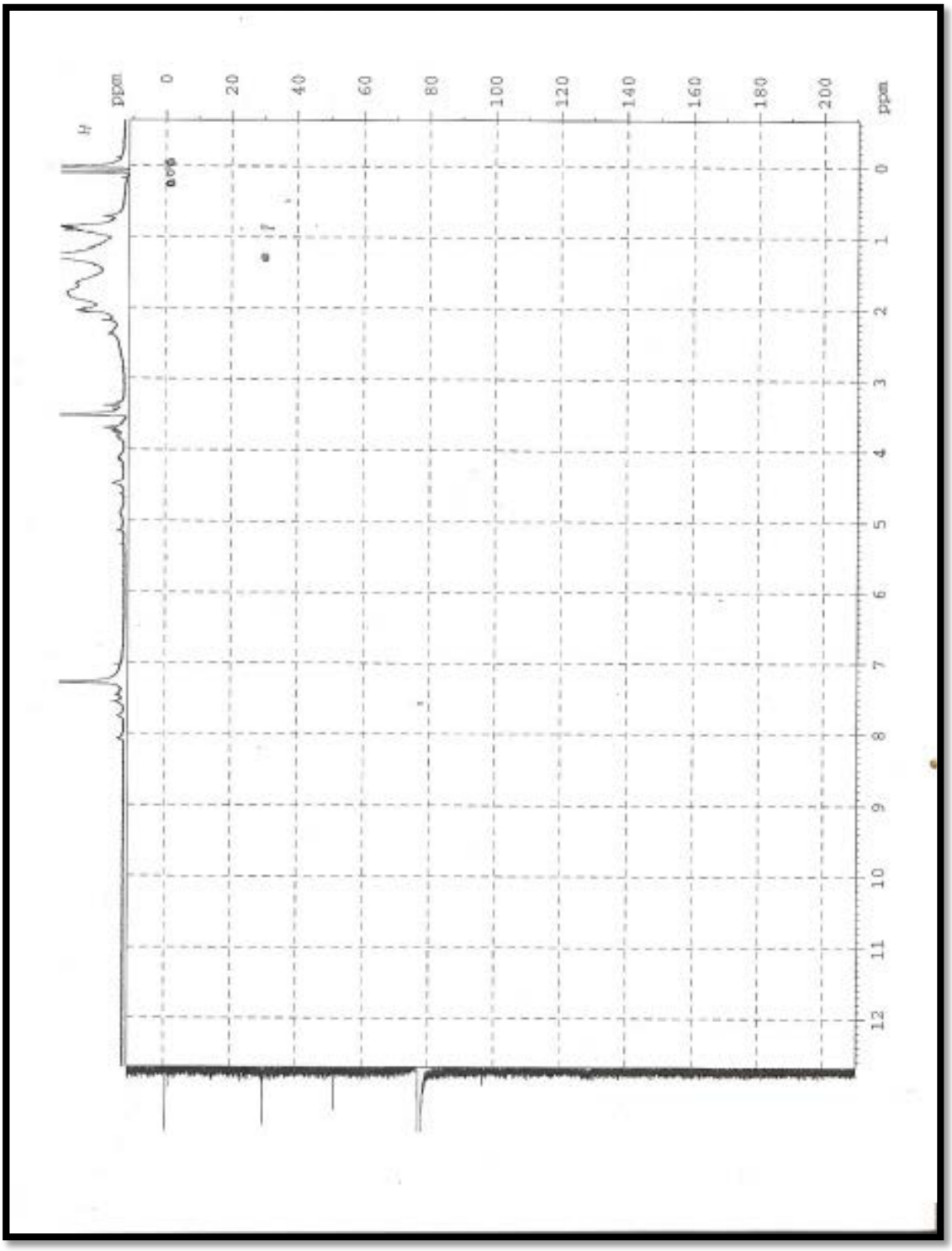


Figure 14. HMBC of KW6-4 in $CDCl_3$.

Table 2. Correlating Carbons and Hydrogens.

Carbon	Carbon shift (ppm)	Proton shift (ppm)	HMBC correlation	COSY correlation
1	170.00	-	-	-
2	22.63	1.87	170.00	2.33
Aliphatic CH_2	30.61	1.75	30.61	-
***C*-O-CH_3**	70.89	3.74	-	-
O-*C*H_3	51.13	3.54	-	-
23	58.49	3.12	-	-

In Figures 9 and 10, both compounds, KW6 and KW6-4, have peaks at 3.54 ppm, which suggests the presence of methoxy groups. Proton shifts ranging from 3.2-3.6 ppm are indicative of methoxy groups in a compound. The peaks at 1.75 ppm indicate the CH_2 groups that would be present in a fatty acid chain. The solvent used was chloroform-*d* which has a peak at 7.2 ppm in the proton 1-D experiment[18].

In Figures 11 and 12, a tall peak at a chemical shift of 30.09 ppm indicated aliphatic CH_2 groups, and peaks at a carbon shift of 51.13 ppm suggested the presence of a methyl group attached to the oxygen in a methoxy group. The ^{13}C spectrum of KW6-4 had a peak at 14.50 ppm that KW6 did not have, which was indicative of a terminal methyl group[18]. It appeared from this data that KW6 did not have a terminal methyl group, or that the amount of sample was too small so the instrument was unable to detect the presence of this functional group. The solvent used for these experiments was again chloroform-*d* which has a peak at 77 ppm in the ^{13}C-NMR experiment[18].

In Figures 13 and 14, the HMBC shows long range correlations between proton and carbons, cross peaks can be detected for more than one carbon. Only few signals were obtained in this 2D experiment.

NMR data compiled together is shown in Table 2. The carboxylic acid with a ppm of 170 was assigned as C1, and it appeared in the HMBC spectrum. Carbon at 22.63 ppm was assigned as C-2 and correlated to protons at 1.87 ppm in HSQC (data not shown). Further, the COSY correlation between 1.87 and 2.33 ppm confirmed this assignment. The carbon with a chemical shift of 70.89 ppm was assigned as the carbon attached to the non-terminal methoxy group and this was shown as a strong correlation in the HMBC but was hidden under the solvent peak in the ^{13}C spectrum. Carbon with a chemical shift of 58.49 was given the assignment of C- 23, and is the terminal carbon attached to a methoxy group, and it correlates to protons with a chemical shift of 3.12 ppm in the HMBC. The CH_2 carbons that comprise the linear aliphatic part of the fatty acid chain had a chemical shift of 30.61 ppm in the carbon spectrum and HMBC. The methoxy carbons (O-CH_3) have a carbon shift of 51.13 and correlate to the protons at 3.54, and did not show in the HSQC (data not shown). All of the NMR experiments performed are 1D ^{1}H-NMR, 1D ^{13}C-NMR, COSY, HMBC, HSQC, NOESY and MS.

Bioassay Data

Table 3. Inhibitory Activity on NF-κB.

Extract	***Activity (%) in 50g Watercress**	***Activity (%) in 1 kg Watercress**
Crude	N/A	20.4
Ethyl Acetate	63	47.3
Hexane	66	46.6
Water	0	11.1

*Rocaglamide was used as the positive control.

Table 4. IC_{50} Values of Isolated Compounds.

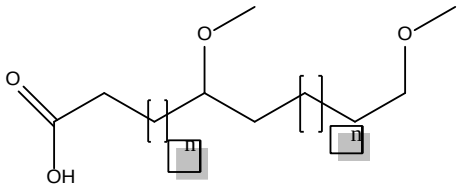
Compound	**IC_{50} Value (μM)**
KW6	Inactive
KW6-4	84
KW6-7	Inactive
Rocaglamide	0.075

Discussion

Three samples, KW6, KW6-4 and KW6-7 were isolated from the hexane extract of watercress. Using HPLC chromatograms and data from MS, it is determined that KW6 and KW6-4 are relatively pure compounds, and KW6-7 is a mixture of compounds. Because sample KW6-7 was determined to be impure through HPLC analysis, no further NMR experiments after the proton NMR (data not shown) were carried out on this sample.

Compounds KW6 and KW6-4 were isolated from a low polarity fraction of the hexane extract (1:9 ethyl acetate: hexanes). The NMR data collected for compound KW6 suggest that it is a fatty acid as it has a signal of 170 ppm in the HMBC. More tests would be needed to determine what kind of compound KW6-4 is as it does not have data that suggest that it is a fatty acid. The two compounds KW6 and KW6-4 have similar molecular weights; 413.26 and 413.28 g/mol, respectively. Because the two compounds are similar in weight, polarity, and their NMR spectra, it is proposed that the two compounds are isomers. They are not the same compound because not only did they both have different retention times (see Figure 3 and Figure 5), but they also had varying activity in the NF-κB assay. It is not uncommon for isomers to have varying biological activity and other different properties.

Using the data from the NMR and MS experiments, a proposed structure of KW6 is below with proposed fragments:



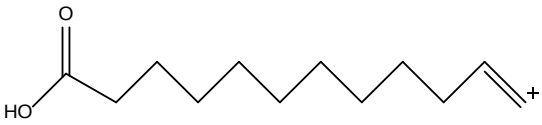
m/z= 413

Proposed Fragments

A.

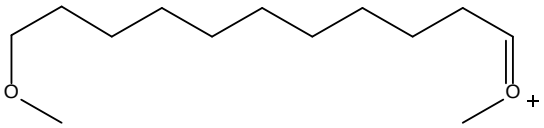
m/z=181

B.

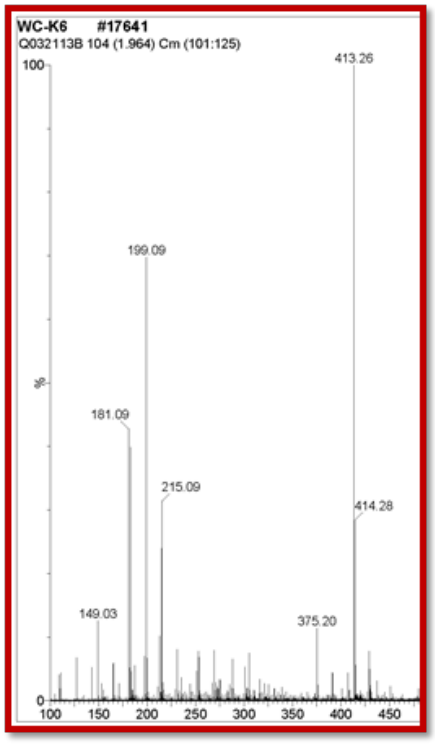


m/z=199

C.



m/z=215



The structure above is the proposed structure of compound KW6 with the location of one of the two methoxy groups unknown, and the other methoxy group located on the terminal carbon of the fatty acid chain because KW6 does not have a terminal methyl signal in its ^{13}C spectrum. It is proposed that KW6-4 looks similar to this structure, except perhaps that it contains a terminal methyl group and the methoxy groups are located elsewhere on the carbon chain. All of the proposed fragments are positively charged and show up in the mass spectrum as shown above. Both KW6 and KW6-4 have peaks at 181, 199 and 215, so these fragments are proposed for both compounds. More experiments would be needed to determine the exact location of the methoxy groups. The proposed elemental chemical formula for KW6 and KW6-4 is $C_{25}H_{49}0_4$. A combustion analysis is needed to confirm the percentages of carbon, hydrogen and oxygen in the compounds.

Watercress contains many isothiocyanates[11], so originally it was assumed that the compounds may contain a sulfur or nitrogen[19]. Comparison of the experimental spectra to the spectra of isothiocyanates[19] led to the conclusion that compounds KW6 and KW6-4 are not isothiocyanates.

For a sample to be considered active, it must show over 50% activity in the NF- κB inhibitory bioassay at a concentration of 50 µg/mL[16]. The hexane and the ethyl acetate extracts of the smaller 50g sample of watercress were active in the assay with 66% and 63% activity respectively, whereas the water extract did not show NF-κB inhibition. The extracts from the larger sample of watercress did not exhibit significant NF-κB inhibition (<50%). Of the three compounds isolated from the hexane extract, KW6-4 was found to be less potent than current anti-inflammatory drugs with an IC_{50} value of 84 µM. This IC_{50} value indicates minimal activity, as IC_{50} values of 20 µM or lower are more desirable.

It is not uncommon for activity to vary in natural products. The activity could be dependent on a number of circumstances: the soil used to enrich the crop, the growing conditions of the crop, how fresh the sample was when it was collected, etc. The extract samples may not have been completely dry at the time of testing, which could have decreased activity as the sample may have been too dilute. Any one of these or another unforeseen factor may have been altered during the time period between the testing of the small and large samples which could result in a difference in activity levels.

Conclusion

The hexane and ethyl acetate extracts from the original 50 g sample of watercress showed promising activity in the NF-κB assay, which is why the experiment was continued with 1 kg watercress. Unfortunately, the activity levels in both the hexane and ethyl acetate are not high enough in the large scale experiment by the criteria used for determining activity. Of the three compounds isolated from the hexane extract, KW6 and KW6-4 are pure and KW6-7 is a mixture. KW6 appears to be a fatty acid derivative and KW6-4 appears to be an isomer of KW6. KW6-4 was the only compound that showed some activity in the bioassay. With drug resistance becoming more and more prevalent in the world, a compound with even weak activity could prove to be useful as a lead candidate. To optimize the activity of KW6-4, the structure can be modified. More compounds could be isolated from watercress that might be responsible for its NF-κB inhibitory activity.

List of Abbreviations

COSY: 2D Correlation Spectroscopy

HMBC: 2D Heteronuclear Multiple Bond Correlation Spectroscopy

HPLC: High Performance Liquid Chromatography

HSQC: 2D Heteronuclear Single Quantum Coherence Spectroscopy

MS: Mass Spectrometry

NF-κB: Nuclear Factor Kappa B

NMR: Nuclear Magnetic Resonance

NOESY: 2D Nuclear Overhauser Effect Spectroscopy

TLC: Thin-Layer Chromatography

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